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FIELD OF THE INVENTION

BACKGROUND OF THE INVENTION

[illegible]

In particular embodiments of the invention, B-cell activation in a subject may be inhibited by contacting helper T cells of the subject with effective amounts of a soluble ligand of CD40CR. Such inhibition of B-cell activation may be especially useful in the treatment of the allergy or autoimmune disease.

One advantage of the present invention is that it enables intervention in an aspect of the immune response which is not antigen specific. Many current therapies for allergy include desensitization to particular antigens, and require that each patient be tested in order to identify antigens associated with sensitivity. As a practical matter, exhaustive analysis of a patient's response to each and every potential allergen is virtually impossible. Furthermore, in most autoimmune conditions, the causative antigen is, generally, unknown or even irrelevant to the disease process. The present invention, which relates to the antigen nonspecific CD40/CD40CR interaction, circumvents the need to characterize the antigen associated with allergy or autoimmunity. Therefore, the present invention may be used to particular advantage in the treatment of allergic conditions in which the immunogen is not known, or has multiple components, for example, in hay fever or in procainamide induced lupus. It may also be useful in acute treatment of immune activation, for example, in therapy for anaphylaxis.

ABBREVIATIONS

Ig	immunoglobulin
mab	monoclonal antibody
PM ^{Act}	plasma membranes prepared from activated helper T-cells
PM ^{rest}	plasma membranes prepared from resting helper T-cells
PAGE	polyacrylamide gel electrophoresis
rIL4	recombinant interleukin 4
rIL5	recombinant interleukin 5

SN	supernatant
T _h	helper T-cell
T _h 1	refers to D 1.6, a I-A ^d -restricted, rabbit immunoglobulin specific clone

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Effect of monoclonal antibodies and CD40-Ig on the induction of B-cell RNA synthesis by PM^{Act}.

Panel A. Resting B-cells were cultured with PM^{rest} or PM^{Act} from T_h1. 25µg/ml of anti-CD4, anti-LFA-1 or anti-ICAM-1 or a combination of each of these (each at 25 µg/ml) was added to wells containing PM^{Act}, and B cell RNA synthesis was measured by incorporation of [³H]-uridine. B-cell RNA synthesis was assessed from 42 to 48 hours post-culture. Results presented are the arithmetic means of triplicate cultures +/- s.d., and are representative of 5 such experiments.

Panel B. Resting B-cells were cultured with PM^{Act} from T_h1 (●, ▲) or T_h2 (□). To the T_h1 PM^{Act} containing culture (●, ▲), increasing amounts of CD40-Ig (▲) or control protein CD7E-Ig (●) were added. To the T_h2 PM^{Act} containing culture (□), increasing amounts of CD40-Ig were added. B-cell RNA synthesis was assessed from 42 to 48 hours post-culture. Results presented are the arithmetic means of triplicate cultures +/- s.d., and are representative of 3 such experiments.

Panel C. Resting B-cells were cultured with LPS (50µg/ml) or PM^{Act}. To cultures, CD40-Ig (25 µg/ml; hatched) or CD7E-Ig (25 µg/ml; solid) were added. RNA synthesis was determined as described in Panel A. Results presented are the arithmetic mean of triplicate cultures +/- s.d., and are representative of 3 such experiments.

Figure 2. CD40-Ig inhibited B-cell differentiation and proliferation.

Panel A. Resting B-cells were cultured with PM^{Act}, rIL4 (10 ng/ml) and rIL5 (5ng/ml). Either at the initiation of culture, or on days 1, 2 or 3 post-initiation of culture, CD40-Ig or CD7E-Ig (25µg/ml) were added. On day six of culture, SN from individual wells were harvested and quantitated for IgM (■) and IgG₁ (●) using an anti-isotype specific ELISA, as described in (Noelle *et al.*, (1991) *J. Immunol.* 146:1118-1124). In the presence of PM^{Act}, IL4 and IL5, (in the absence of added CD40-Ig) the concentrations of IgM and IgG₁ were 4.6 µg/ml and 126 ng/ml, respectively. Cultures which received CD7E-Ig (25 µg/ml) on Day 0 produced 2.4 µg/ml and 89 ng/ml of IgM and IgG₁ respectively. In the absence of IL4 and IL5, no IgM or IgG₁ was detected. Results are representative of 3 such experiments.

Panel B. T_h1 were rested or activated with anti-CD3 for 16 hours, irradiated and cultured (1x10⁴/well) with resting B-cells (4X10⁴/culture) in the presence of IL4 (10 ng/ml). Between 0 and 25 µg/ml of CD40-Ig (▲) or CD7E-Ig (●) were added to cultures. From 66-72 hours post-culture, wells were pulsed with 1.0 µCi of [³H]-thymidine and harvested. The dotted line indicates the response of B-cells to resting T_h. Results presented are the arithmetic mean of triplicate cultures +/- s.d., and are representative of 2 such experiments.

Figure 3. CD40-Ig detected a molecule expressed on activated, but not resting T_h. Resting and activated, T_h were harvested and incubated with fusion proteins for 20 minutes at 4°C, followed by FITC-conjugated goat anti-hIgG (25 µg/ml). Percentage positive cells and MFI were determined by analysis of at least 5000 cells/sample. Results are representative of 6 such experiments. CD40-Ig binding is indicated by a filled-in profile.

Figure 4. CD40-Ig immunoprecipitated a 39 kD protein from lysate of activated T_h1. T_h1 were rested or activated with insolubilized anti-CD3 for 16 hours. [³⁵S]-labelled proteins from resting or activated T_h were immunoprecipitated with purified antibodies or fusion proteins (1-10µ). The gel profile is representative of 3 such experiments.

Figure 5. A monoclonal antibody (mab), specific to the induced 39 kD T_h membrane protein, inhibited induction of B-cell RNA synthesis by PM^{Act}. Resting B-cells and PM^{Act} were cultured with 10 µg/ml each of anti-α/β, anti-CD3, CD40-Ig or MR1. RNA synthesis was

For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

- (i) ligands that bind to CD40CR;
- (ii) methods used to characterize CD40CR;
- (iii) preparation of purified CD40CR;
- (iv) uses of ligands that bind to CD40CR;
- and
- (v) uses of CD40CR.

Ligands that Bind to CD40CR

The present invention provides for soluble ligands of CD40CR, including (i) fusion molecules comprising at least a portion of CD40 protein and (ii) antibodies or antibody fragments.

The term “soluble”, as used herein, indicates that the ligands of the invention are not permanently associated with a cell plasma membrane. Soluble ligands of the invention may, however, be affixed to a non-cellular solid support, including a lipid, protein, or carbohydrate molecule, a bead, a vesicle, a magnetic particle, a fiber, etc. or may be enclosed within an implant or vesicle.

The ability of such a ligand to bind to CD40CR may be confirmed by demonstrating that the ligand binds to the same protein as CD40-Ig (infra) or MRI (infra).

The ligands of the invention may be comprised in pharmaceutical compositions together with a suitable carrier.

Fusion Molecules

The present invention provides for soluble fusion molecules that are ligands of CD40CR. Such fusion molecules comprise at least a portion of CD40 protein attached to a second molecule. The portion of CD40 preferably lacks the CD40 transmembrane domain. A portion of CD40 protein which may be used according to the invention is defined as any portion which is able to bind to CD40CR, for example, such a portion may be shown to bind to the same protein as MR1 or CD40-Ig.

Second molecules which may be used include peptides and proteins, lipids, and carbohydrates, and, in preferred embodiments of the invention, may be an immunoglobulin molecule, or portion thereof (such as an Fv, Fab, F(ab')₂, for Fab' fragment) or CD8, or another adhesion molecule, such as B7. The second molecule may be derived from either a non-human or a human source, or may be chimeric. The second molecule may also be an enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound.

The fusion molecules of the invention may be produced by chemical synthesis or, preferably, by recombinant DNA techniques.

For example, a nucleic acid sequence encoding at least a portion of CD40 protein may be combined with a nucleic acid sequence encoding a second molecule in a suitable expression vector, and then expressed in a prokaryotic or, preferably, eukaryotic expression system, such as a yeast, baculovirus, or mammalian expression system, including transgenic animals.

Alternatively, at least a portion of CD40 protein may be expressed using recombinant DNA techniques and then may be chemically conjugated to a second molecule.

Fusion molecules comprising CD40 may be purified from preparative mixtures using electrophoretic techniques or affinity chromatography using ligand that binds to either CD40 or to the second molecule. Ligands that bind to CD40 include, but are not limited to, anti-CD40

antibodies such as G28-5, as produced by the hybridoma having accession number HB9110 and deposited with the American Type Culture Collection, and CD40CR, described more fully infra. If the second molecule is an immunoglobulin or immunoglobulin fragment, an affinity column comprising anti-immunoglobulin antibody may be used; if the second molecule comprises an F_c fragment, a protein A column may be used.

According to a preferred embodiment of the invention, a portion of CD40 may be produced using a nucleic acid sequence that encodes a CD40 protein that is truncated upstream from the transmembrane domain. Such a nucleic acid sequence may be prepared by digesting a plasmid containing cDNA encoding CD40 antigen, such as that described in *Stamenkovic et al.*, (1989), *EMBO J.* 8:1403-1410, with PstI (P) and Sau 3A (S3) restriction enzymes. The resulting P/S3 fragment may be subcloned into the same plasmid digested with P and Bam HI (B), to produce a truncated CD40 gene (see Figure 8).

In particular, nonlimiting, embodiments of the invention, an expression vector used to produce ligands containing at least a portion of CD40 as well as immunoglobulin sequence may preferably comprise a virally-derived origin of replication, a bacterial origin of replication, a bacterial selectable marker, and eukaryotic promoter and enhancer sequences separated from DNA sequences encoding an immunoglobulin constant region by restriction endonuclease sites which allow subcloning of DNA sequences encoding at least a portion of CD40, followed by a polyadenylation signal sequence (see Figure 8.b.).

In a specific embodiment of the invention, the truncated CD40 gene may be subcloned into an immunoglobulin fusion plasmid, such as that described in *Aruffo et al.*, 1990, *Cell* 61:1303-1313, using an Mlu I and B digest, to form plasmid pCD40-Ig, which encodes the fusion molecule CD40-Ig (see Figure 8). CD40-Ig fusion protein may then be produced by transfecting the pCD40-Ig plasmid into COS cells to form a transient expression system. CD40-Ig produced may be collected from the COS cell supernatant and purified by protein A column chromatography as described in *Aruffo et al.*, 1990, *Cell* 161:1303-1313.

Antibodies

The soluble ligands of the invention may comprise antibody molecules, monoclonal antibody molecules, or fragments of these antibody molecules which contain an antigen combining site that binds to CD40CR. Such ligands may further comprise a second molecule which may be protein, lipid, carbohydrate, enzyme, toxin, growth factor, lymphokine, antiproliferative agent, alkylating agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound and may be linked to the antibody molecule or fragment.

Where the ligand is a monoclonal antibody, or a fragment thereof, the monoclonal antibody can be prepared against CD40CR using any technique which provides for the production of antibody molecules by continuous cell lines in culture. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, *Nature* 256:495-497) as well as other techniques which have more recently become available, such as the human B-cell hybridoma technique (Kozbar et al., 1983, *Immunology Today* 4:72) and EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96) and the like are within the scope of the present invention.

Antibody fragments which contain the idiotype of the molecule could be generated by known techniques. For example, such fragments include but are not limited to: the $F(ab')_2$ fragment which can be generated by treating the antibody molecule with pepsin; the Fab' fragments which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragment; the $F(ab')_2$ fragment which can be generated by treating the antibody molecule with papain; and the 2Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent to reduce the disulfide bridges.

The present invention also provides for chimeric antibodies produced by techniques known in the art, such as those set forth in *Morrison et al.*, (1984) *Proc. Natl. Acad. Sci. U.S.A.* 81:6851-6855 or *European Patent Application No. 85305604.2*, publication No. 0173494 by *Morrison et al.*, published March 5, 1986.

Immunogen for the production of antibodies may be any source that contains CD40CR. For example, activated T_h may be used as an immunogen.

Alternatively, substantially purified CD40CR, prepared as set forth ~~in section 5.3,~~ infra, may be used. If activated T_h are used as immunogen, antiserum may be tested for reactivity against activated but not resting T_h cells.

In a preferred embodiment of the invention, the soluble ligand is the MR1 monoclonal antibody. The following method was used to produce the MR1 monoclonal antibody, and may be used to generate other antibodies directed toward CD40CR.

Hamsters were immunized intraperitoneally with 5-10⁶ activated T_h1 cells (D1.6) at weekly intervals for six weeks. When the serum titer against murine T_h1 was greater than about 1:10,000, cell fusions were performed with polyethylene glycol using immune hamster splenocytes and NSI. SN from wells containing growing hybridomas were screened by flow cytometry on resting and activated T_h1. One particular hybridoma, which produced a mab that selectively recognized activated T_h, was further tested and subcloned to derive MR1. MR1 was produced in ascites and purified by ion exchange HPLC.

The present invention also provides for ligands comprising monoclonal antibodies, and fragments thereof that are capable of competitively inhibiting the binding of MR1 to its target antigen or CD40-Ig to its receptor.

Methods Used to Characterize CD40R

CD40CR may be characterized by (i) its ability to bind CD40, fusion molecules comprising at least a portion of CD40, and antibodies such as MR1; (ii) its functional characteristic of being able to stimulate B-cell cycle entry, proliferation, and differentiation; and (iii) its cellular distribution.

CD40CR may be characterized by its ability to bind to ligands such as CD40, fusion molecules comprising CD40, and antibodies directed toward CD40CR.

As discussed in greater detail infra, several techniques were used to characterize CD40CR. For example, CD40-Ig and MR1 were shown to recognize the same 39 kD molecule. Both CD40-Ig and MR1 were found to immunoprecipitate a 39 kD protein from radiolabelled T_h lysates (Figure 5b). Further, immunoprecipitation of the 39 kD protein with CD40-Ig removed the antigen recognized by MR1 from T_h lysates.

CD40CR may also be characterized by its ability to stimulate B-cell cycle entry, proliferation, and differentiation.

For example, plasma membrane (PM) from activated (PM^{Act}) but not resting (PM^{rest}) T_H cells was found to induce B-cell RNA synthesis (Figure 1a); this induction, indicative of B-cell activation, was not; affected by antibodies such as anti-LFA-1, anti-CD4, anti-ICAM-1. CD40-Ig or MR1, however, were found to be able to inhibit PM^{Act} -induced B-cell activation, as shown in Figure 1b and Figure 6.

The induction of B-cell activation may be measured by techniques such as [³H]-uridine incorporation into RNA (as B-cells differentiate, RNA synthesis increases), or by [³H]-thymidine incorporation, which measures DNA synthesis associated with cell proliferation. For optimal measurement of the effect of CD40CR on B-cell proliferation, interleukin-4 (IL-4) may be added to the culture medium at a concentration of about 10 ng/ml.

Alternatively, B-cell activation may be measured as a function of immunoglobulin secretion. For example, CD40Cr, in substantially purified form, or as present in PM, or otherwise, may be added to resting B-cells together with IL-4 (10 ng/ml) and IL-5 (5 ng/ml). After three days of culture, an additional volume of culture medium may be added. On a day 6 of culture, supernatant (SN) from individual cultures may be harvested and quantitated for IgM and Ig₁ as described in Noelle *et al.*, (1991) *J. Immunol.* 146:1118-1124.

CD40CR may also be characterized by its cellular distribution. For example, CD40-Ig was observed to bind to activated, but not resting T_h1, as assessed by flow cytometry (Figure 3). Furthermore, CD40-Ig was observed to bind to Jurkat cells, HSB2 cells, and activated T-

agent, antimetabolite, antibiotic, vinca alkaloid, platinum coordinated complex, radioisotope, or a fluorescent compound.

The present invention further provides for substantially purified CD40CR which has been prepared by chemical synthesis or recombinant DNA techniques. For example, the gene for CD40CR may be isolated by inserting cDNA prepared from activated helper T-cells into the λ gt10 expression system, and then screening with MR1 or CD40-Ig binding to identify CD40CR-expressing clones. Alternatively, cDNA prepared from activated helper T-cells may be transfected into COS cells, the supernatants of which may be screened with MR1 or CD40-Ig to identify CD40CR producers. The gene for CD40CR may be then used to express CD40CR using expression systems in the art.

Uses of Ligands that Bind to CD40CR

The present invention provides for methods of controlling B-cell activation that utilize ligands that bind to CD40CR. In particular, it provides for a method of inhibiting B-cell activation comprising exposing a mixture of B-cells and T_h cells to an effective concentration of ligand that binds to CD40CR. Ligands that may be used are described supra. The method of the invention may be practiced in vitro or in vivo. An effective concentration refers to a concentration of a ligand that inhibits B-cell activation, measured by any technique known in the art (including those set forth supra) by at least about 30 percent, and preferably by about 75 percent. According to a preferred, specific, non-limiting embodiment of the invention, CD40-Ig may be used as ligand, in which invention, CD40-Ig may be used as ligand, in which case an effective concentration may be at least about 10 μ g/ml. In another specific, nonlimiting embodiment of the invention, the monoclonal antibody MR1 may be used, in which case an effective concentration may be at least about 10 μ g/ml. If the method is practiced in vivo, an effective concentration of ligand may refer to plasma concentration of ligand or to a local concentration. For example, it may be desirable to inhibit B-cell activation in a localized area in order to limit the effects on the immune system as a whole.

In particular embodiments, the invention provides for a method of treating a subject suffering from a disorder associated with B-cell activation, comprising administering to the

subject a therapeutic amount of ligand that binds to CD40CR. A subject may be a non-human or, preferably, a human animal.

Disorders associated with B-cell activation include, but are not limited to, allergy (including anaphylaxis); autoimmune conditions including drug induced lupus, systemic lupus erythematosus, adult rheumatoid arthritis, juvenile rheumatoid arthritis, scleroderma, Sjogren's Syndrome, et c.; and viral diseases that involve B-cells, including Epstein-Barr infection, and retroviral infection including infection with a human immunodeficiency virus.

Because it has been suggested that B-cell activation is associated with the induction of human immunodeficiency virus replication from latency, it may be desirable to administer the ligands of the invention to HIV positive individuals who have not yet developed AIDS or ARC.

Ligands may be administered, in a suitable pharmaceutical carrier, by any method known in the art, including intravenous, intraperitoneal, subcutaneous, intrathecal, intraarticular or intramuscular injection, and oral, intranasal, intraocular and rectal administration, and may be comprised in microspheres, liposomes, and/or sustained released implants.

A therapeutic amount of ligand is defined as an amount which significantly diminishes the deleterious clinical effects of B-cell activation, and may vary among ligands used and conditions treated. If CD40-Ig is used, therapeutic concentration may be about 10 µg/ml either systemically (plasma concentration) or locally. If MR1 is used, a therapeutic concentration may be about 10 µg/ml either systemically (plasma concentration) or locally.

In a further embodiment of the invention, the above methods may utilize a ligand comprising a toxin or antimetabolite such that T_h cells are killed or damaged and B-cell activation is decreased as a result of T_h cell destruction.

The ligands of the invention may also be used to label activated T cells, a technique which may be useful in the diagnosis of T cell disorders. To this end, ligand comprising an

enzyme, radioisotope, fluorescent compound or other detectable label may be exposed to T cells in vitro or in vivo and the amount of binding may be quantitated.

The ligands of the invention may also be used to deliver substances, e.g. growth factors, to be activated T-cells.

Uses of CD40CR

The present invention provides for methods of controlling B-cell activation that utilize CD40CR or a molecule comprising CD40CR, prepared as described supra. In particular, it provides for a method of promoting B-cell activation comprising exposing B-cells to an effective concentration of CD40CR. The method may be practiced in vivo or in vitro. An effective concentration refers to a concentration of receptor that induces B-cell activation, measured by any technique known in the art (including those set forth supra) by at least about 30 percent. In specific, nonlimiting embodiments of the invention, the concentration of CD40CR may be about 10 µg/ml locally or systemically.

In particular embodiments, the invention provides for a method of treating a subject suffering from an immunodeficiency disorder associated with diminished humoral immunity, comprising administering to the subject a therapeutic amount of CD40CR. A subject may be a non-human or, preferably, a human animal.

Immunodeficiency disorders associated with diminished humoral immunity include acquired immunodeficiency syndrome, immunodeficiency associated with malignancy or cachexia, iatrogenic immunodeficiency caused, for example, by chemotherapy or radiation therapy, as well as genetic disorders involving humoral immunity.

CD40CR may be administered, in a suitable pharmaceutical carrier, by any method known in the art, including intravenous, intraperitoneal, subcutaneous, intrathecal, intraarticular, or intramuscular injection, and oral, intranasal, intraocular, and rectal administration and may be comprised in micropsheres, liposomes, and/or sustained release implants.

A therapeutic amount of CD40CR for CD040 is defined as that amount which increases immunoglobulin production by at least about 30 percent.

In a further embodiment, CD40CR may be conjugated to a toxin, and then administered to a subject under circumstances in which it would be preferable to destroy B-cells that express CD40. Examples of such circumstances include patients receiving organ transplants or suffering from multiple myeloma or another B-cell malignancy, or from autoimmune disease.

CD40CR may also be used to label B-cells expressing CD40, a technique which may be useful in the diagnosis of B-cell disorders. To this end, receptor linked to an enzyme, radioisotope, fluorescent compound or other detectable label may be exposed to B-cells in vivo or in vitro and the amount of binding may be quantitated.

CD40CR may also be used to deliver molecules that are linked to it to B-cells.

EXAMPLES

Example 1: A Novel Receptor, CD40CR, On Activated Helper T-Cells Binds CD40 And Transduces the Signal for Cognate Activation of B-Cells

Materials And Methods

Animals

Female DBA/2J mice (Jackson Laboratories, Bar Harbor, MA) were used for the preparation of filler cells to support the growth of T_h clones and in the preparation of resting B-cells.

Helper T-Cell Clones (T_h)

D1.6, a I-A^d-restricted, rabbit Ig-specific T_h1 clone (*Kurt-Jones et al., (1987), J Exp Med 166:1774-1787*) was obtained from Dr. David Parker, University of Mass. at Worcester. D1.6 will be referred to herein as T_h1.

Activation of Th by Anti-CD3

Th1 were cultured (8×10^6 /well) in cluster wells (6 well, Corning, NY) coated with 40 μ g/4 ml of PBS/well with anti-CD3 for 16 hours, as described in (Noelle *et al.*, (1991) *J. Immunol.* 146:1118-1224).

Preparation of Th Plasma Membranes

Plasma membranes were prepared by discontinuous sucrose gradient sedimentation, as described in (Noelle *et al.*, (1991) *J. Immunol.* 146:1118-1124).

Preparation of Resting B Cells

Resulting splenic B-cells were prepared by sedimentation on discontinuous PERCOLL[®] gradients, as described (Defrango *et al.*, (1982) *J. Exp. Med.* 155:1523). Cells isolated from the 70-75% (density of 1.087-1.097) PERCOLL[®] interface were typically >95% mIg⁺, had a uniform, low degree of near forward light scatter and were unresponsive to Con A.

Antibodies

The following mabs were purified by ion exchange HPLC from ascites fluid of mice which had been irradiated and bone marrow reconstituted: anti-CD3:145-2C11 (Leo *et al.*, (1987) *Proc. Natl. Acad. Sci. USA* 84: 1374-1378); anti- α,β :H57-597; anti-CD4: GK1.5 (Wilde *et al.*, (1983) *J. Immunol.* 131:2178-2183); anti-ICAM: YN1/1.7.4 (Prieto *et al.*, (1989) *Eur. J. Immunol.* 19:1551-1557); anti-LFA-1: FD441.8 (Sarmiento *et al.*, (1982) *Immunol. Rev.* 68:135); and anti-rat/hamster κ chain:RG-7 (Spring, (1982) *Hybrid.* 1:257-273).

Preparation of the CD40 Recombinant Globulin (CD40-Ig)

The CD40 fusion protein was prepared by digesting a plasmid containing a cDNA encoding the CD40 antigen (Stamenkovic and Seed, (1989) *EMBO J.* 8:1403-1410) with the restriction enzyme Pst I (P) and Sau 3A (S3). This P/S3 fragment was subcloned into the same plasmid digested with P and Bam H1 (B). This allowed the preparation of the CD40 which encoded a CD40 protein truncated upstream from the transmembrane domain. The DNA

fragment encoding a CD40 α was then subcloned into the immunoglobulin fusion plasmid (Aruffo et al. (1990), Cell. 61:1301-1313) using a MluI and B digest. The CD40-Ig fusion protein was produced by transient transfection in COS cells and purified on a protein A column as described in (Aruffo et al., (1990) Cell. 61:1303-1313).

Lymphokines

Interleukin 4 (IL4): Recombinant mouse IL4 was generously provided by Drs. C. Maliszewski and K. Grabstein, Immunex Corporation, Seattle, WA.

Interleukin 5 (IL5): Recombinant mouse IL5 was purchased from R&D Research, Sarrento, CA.

Induction of B-Cell RNA Synthesis by Activated Th Plasma Membranes

3 X 10⁴ resting B-cells were cultured in 50 μ l of cRPMI in A/2 microtiter wells (Costar, Cambridge, MA). To these wells, 0.5 μ g of T_h1 or T_h2 membrane protein was added. From 42-48 hrs, wells, were pulsed with 2.5 μ Ci of ³H-uridine (New England Nuclear, Boston MA), harvested, and the radioactivity determined by liquid scintillation spectroscopy. The results were expressed as cpm/culture +/-s.d.

Induction of B-Cell Immunoglobulin Secretion by Activated Th Plasma Membranes and Lymphokines

Resting B-cells were cultured as described above. To culture wells, 0.5 μ g of T_h1 membrane protein, IL4 (10 ng/ml) and IL5 (5 ng/ml) were added. On day three of culture, an additional 50 μ l of cRPMI was added. On day three of culture, an additional 50 μ l of cRPMI was added. On day six of culture, SN from individual wells were harvested and quantitated for IgM and IgG₁, as described in (Noelle et al., (1991) J. Immunol. 146:1118-1124).

Induction of B-Cell Proliferation by Activated Th and IL4

4×10^4 resting B-cells were cultured in 50 μ l of cRPMI in A/2 microtiter wells (Costar, Cambridge, MA). To these wells, 1×10^4 resting or activated, irradiated (500 rads) Th1 and Il4 (40 ng/ml) were added. On day three of culture, wells were pulsed with 1 μ Ci of 3 H thymidine, as described in (Noelle et al., (1991) *J. Immunol.* 146:1118-1124).

Production of Monoclonal Antibodies Specific to Membrane Proteins Induced on Activated Th1

Hamsters were immunized intraperitoneally with $5-10 \times 10^6$ activated Th1 (D1.6) at weekly intervals for six weeks. When the serum titer against murine Th1 was greater than 1:10,000, cell fusions were performed with polyethylene glycol using immune hamster splenocytes and NS1. SN from wells containing growing hybridomas were screened by flow cytometry on resting and activated Th1. One particular hybridoma, which produced a mab that selectively recognized activated Th, was further tested and subcloned to derive MR1. MR1 was produced in ascites and purified by ion exchange HPLC.

Flow Cytofluorometric Analysis of Activation Molecules Expressed on Th

Resting and activated Th (16 hours with anti-CD3) were harvested and incubated at 1×10^5 cells/50 μ l with fusion protein for 20 minutes at 4°C, followed by FITC-conjugated goat anti-human (h)IgG (25 μ g/ml; Southern Biotechnology, Birmingham, AL). To all samples, propidium iodide was added at final concentration of 2 μ g/ml. Flow cytofluorometric analysis was performed on a BD FACSCAN. After positive gating of cells by forward versus side scatter, and by red negativity (for propidium iodide exclusion), the log green fluorescence of viable cells was ascertained. At least 5,000 viable cells were analyzed for the determination of percent positive cells and MFI. Staining with MR1 employed FITC-conjugated RG7, a mouse anti-rat/hamster κ chain mab.

Biosynthetic Labelling, Immunoprecipitation, SDS-PAGE and Fluorography

T_h1 were rested or activated with insolubilized anti-CD3 for 16 hrs. Proteins from resting and activated T_h1 (20 x 10⁶/ml) were labelled with 1 mCi of [³⁵S]-methionine/cysteine for one hour, at which time they were washed twice in RPMI/10%FCS and the cell pellet was lysed in extraction buffer, as described (Noelle *et al.*, (1991) *J. Immunol.* 146:1118-1124). Purified antibodies or fusion proteins (1-10 µg) were added to 500ul of lysate (5x10⁶ cell equivalents) at 4°C for 16 hours. At that time, the lysates were transferred to tubes containing 50 µl of packed Protein A-sepharose. The pelleted Protein A-Sepharose was resuspended and tubes were incubated at 4°C for 1 hr with agitation. The samples were then washed 3x with high stringency wash buffer. The pelleted protein A-Sepharose was resuspended in 30 µl of SDS sample buffer and run on a 10% polyacrylamide gel. After running the gel, the gel was fixed and fluorography performed.

Results

Effect of Monoclonal Antibodies on the Induction of B Cell RNA Synthesis by PM^{Act}

In order to define the cell surface molecules that mediated the induction of B-cell cycle entry by PM^{Act}, mabs to T_h membrane proteins were added to cultures of PM^{Act} and B-cells. PM^{Act} induced B-cell RNA synthesis eight-fold over that observed with PM^{rest} (Figure 1a). The addition of anti-LFA-1, anti-CD4, anti-ICAM-1, alone, or in combination, did not inhibit the induction of B-cell RNA synthesis by PM^{Act}.

CD40-Ig Inhibited T-Induced B-Cell Cycle Entry, Differentiation and Proliferation

In the human system, it had been shown that anti-CD40 mab induced B-cell proliferation (Clark and Lane, (1991) *Ann. Rev. Immunol.* 2:97-127) thereby implicating CD40 as an important triggering molecule for B-cells. To determine if CD40 was involved in the induction of B-cell RNA synthesis by PM^{Act}, a soluble fusion protein of the extracellular domains of human CD40 and the F_c domain of human IgG₁ (CD40-Ig) was added to cultures of PM^{Act} derived from T_h1 and T_h2 were prepared and used to stimulate B-cell RNA synthesis.

CD40-Ig Detected a Molecule Expressed on Activated, but not Resting Th

To investigate whether activated Th1 express a binding protein for CD40, resting and activated (16 hours) Th1 were stained with CD40-Ig or CD7E-Ig, followed by FITC-anti-HigG. Binding of CD40-Ig was assessed by flow cytometry (Fig. 3). Th1 that were activated for 16 hours with anti-CD3, but not resting Th1, stained 56% positive with CD40-Ig, but not with the control CD7E-Ig. To identify the CD40-Ig binding protein, Th1 proteins were biosynthetically labelled with [³⁵S]-methionine/cysteine and proteins immunoprecipitated with CD40-Ig or CD7E-Ig. The immunoprecipitated proteins were resolved by SDS-PAGE and fluorography (Figure 4). A prominent band with an apparent molecular weight of 39 kD immunoprecipitated in a dose-dependent manner with 1 and 10 µg of CD40/sample. As controls, anti-class I mab immunoprecipitated bands at 55 kD and a low molecular weight band, β2 microglobulin. In the absence of mab, no prominent bands were visible. A 39 kD band was also immunoprecipitated from activated Th that were vectorially labelled with ¹²⁵I, confirming that the 39kD protein was a membrane protein.

Monoclonal Antibody MR1, Specific to 39Kd Th Membrane Protein Inhibited the Induction of B-Cell RNA Synthesis by PMAct

Mabs specific to antigens selectively expressed on activated versus resting Th were developed to identify Th molecule(s) responsible for the Th effector phase activity. One such mab, MR1, recognized an antigen that was selectively expressed on activated Th1. To investigate whether MR1 and CD40-Ig recognized the same molecule, flow cytometry and blocking studies were performed. CD40-Ig and MR1 stained approximately 56% and 61%, respectively, of activated, but not resting Th (Fig. 5a). MR1, but not another hamster anti-T cell mab, anti-α/β TCR, blocked the staining of activated Th1 with CD40-Ig, in a dose-dependent manner. These data suggested that CD40-Ig and MR1 recognized overlapping or identical epitopes on the 39 kD Th protein. To further demonstrate that CD40-Ig and MR1 recognized the same molecule, the antigen that bound MR1 was identified by immunoprecipitation of proteins from radiolabelled Th lysates. Both CD40-Ig and MR1 immunoprecipitated a 39 kD protein (Fig. 5b). Finally, immunoprecipitation of the 39kD

protein with CD40-Ig removed the antigen recognized by MR1 from radiolabelled lysates of activated T_h supporting the tenet that MR1 antigen and the CD40 binding protein were identical.

Functional studies were performed with MR1 to address whether theis mab neutralized the activity expressed by PM^{Act}. PM^{Act} and B-cells were cultured alone, or in the presence of hamster mabs or CD40-Ig. Two hamster mabs, anti- α/β TCR and α -CD3 did not inhibit the activation of resting B-cells by PM^{Act}. In contrast, MR1 or CD40-Ig inhibited B-cell activation (Fig. 6).

Discussion

The data show that blocking of prominent T_h surface molecules (LFA-1, CD4, ICAM-1, CD3, α/β TCR) with mabs did not impede the capacity of activated T_h to induce B-cell cycle entry. In contrast, CD40-Ig or a maB specific to the CD40 binding protein, blocked T_h-dependent B-cell activation in a dose-dependent manner. Furthermore, the CD40 binding protein was identified as a 39 kD protein that is selectively expressed on the membranes of activated, but not resting T_h. Both CD40-Ig and a mab specific to the 39kD CD40 binding protein blocked B-cell activation by PM^{Act}.

Although a number of membrane proteins have been implicated in T_h-dependent B-cell signalling, evidence presented herein dismisses the contribution of some molecules (LFA-1, CD4, CD3, α/β TCR, ICAM-1) and implicates CD40 as the B-cell receptor for cognate signalling by T_h. Data show that CD40-Ig and a mab specific to the CD40 binding protein inhibits T_h-dependent B-cell activation.

The ligand for CD40 is a 39 kD protein that is expressed on activated, but not resting T_h. Biochemical studies indicate that the 39 kD protein is a single chain molecule since electrophoretic migration was not influenced by reducing agents. Based on the functional studies presented in this study, both activated T_h1 and T_h2 express the 39 kD CD40 binding protein. This is consistent with the functional studies that show both T_h1 and T_h2 induce B-cell cycle entry. In an attempt to further characterize the 39 kD protein, cDNA encoding CD

public of the hybridoma cell line MR1 will be irrevocably withdrawn upon issuance of a United States Patent to this application. Also, access to the MR1 cell line will be available to the Commissioner during the pendency of this patent application or to one determined by the Commissioner to be entitled to such cell line under 37 C.F.R. §1.14 and 35 U.S.C. §122.